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Engineered Rhizosphere: the Trophic Bias Generated by Opine-Producing Plants Is Independent of the Opine Type, the Soil Origin, and the Plant Species

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In a previous study, we demonstrated that transgenic *Lotus* plants producing opines (which are small amino acid and sugar conjugates) specifically favor growth of opine-degrading rhizobacteria. The opine-induced bias was repeated and demonstrated with another soil type and another plant species (*Solanum nigrum*). This phenomenon is therefore independent of both soil type and plant species.

The use of microorganisms as biopesticides or plant growth enhancers is an attractive alternative to the use of chemical pesticides and fertilizers (3, 10, 12, 35, 37). However, introduction of plant-growth-promoting bacteria in open fields often fails. This is attributed to limited survival of the inoculant strain in the rhizosphere, where it faces competition from resident microorganisms, a diverse community well adapted to the biological and physicochemical properties of the plant-soil interface (37). It is therefore crucial to develop methods to extend the fitness and persistence of the inoculant microorganisms, possibly by introducing a bias in the competition that benefits the isolate inoculated (20). This bias may be generated by addition to the soil, or release by the plant, of one or more substrates utilizable only by the introduced strain. This approach has been successfully used to sustain growth of various microbes in soil (1, 2, 5). Similarly, plants engineered to produce bacterial growth substrates have been shown to specifically select populations of microbes utilizing these substrates in the rhizospheres of *Lotus* (8, 21) and tobacco plants (31). Most often, these growth substrates have been opines (4), a family of compounds derived from amino acids and/or sugars and specifically detected in the crown gall tumors and hairy root formations induced by members of the genus *Agrobacterium* (4).

Bacterial populations are highly dependent upon soil type (13, 14, 24, 23, 32) and plant exudates (7, 15, 17, 38). Therefore, there is a risk that a selective microbial substrate strategy might be successful for a single soil type or a single plant species or cultivar. The work described here was aimed at determining whether the impact of opine production on soil bacteria is independent of the type of opines produced by the plant, the origin of the soil, and the plant species producing the opines. Such investigations are crucial to evaluate whether opine-producing plants and biased rhizosphere strategies could be used to engineer plant-microbe interactions under various conditions.

To address the questions above, plants of the legume *Lotus corniculatus* cv. Rodéo and *Solanum nigrum* plants were engineered via *Agrobacterium rhizogenes* transformation to produce opines, as described by Petit et al. (26). The transformed plants produced the opines mannopine, mannopine and nopaline, or mannopine and octopine; the latter opine has not been tested previously (Fig. 1) (for a review, see reference 4). Transformed control plants harboring the pRi oncogenes but producing no opines (ONC plants) were also generated by using the same procedure (26). Plants were increased by propagating cuttings for 3 to 4 weeks on Murashige-Skoog medium (catalog no. M 11225; Sigma France, L'Isle d'Abeau, France) supplemented with sucrose (20 g/liter) and 0.5× Morel-Wetmore vitamin mixture (18) at 23°C under long-day conditions (16 h of light per day) to a stage that allowed transfer to a greenhouse (6 to 10 cm long for *Lotus* plants, four to six leaves for *S. nigrum*). Once transferred, plants were grown for up to 18 weeks under long-day conditions (16 h of light per day; 24°C during the day and 17°C at night) in a nonsterilized soil from La-Côte-Saint-André (a loamy-sandy soil from Isère, France), which differed from the soil from La Mérantaise (a loamy, clay-rich soil from Essonne, France) used in previous studies (21, 22). For each experiment, three microcosms, each containing three or four plants of the same line (wild-type [WT], ONC, or opine-producing plants), were set up.

The bacterial populations of the rhizospheres were recovered and analyzed as indicated by Oger et al. (21, 22). Total cultivable bacterial populations and the fluorescent *Pseudomonas* populations were counted on modified Luria-Bertani medium (which contained 5 g of NaCl per liter instead of 10 g/liter) at 28°C and on King's B medium (11) at 25°C, respectively. Fluorescence of colonies was assessed under UV light at 365 nm. Both media were supplemented with cycloheximide (250 mg/liter). The densities of cultivable organisms that utilized opines (mannopine, nopaline, and octopine) were evaluated by inoculating 50 μl of AT minimal medium (25) supplemented with the appropriate opine(s) (5 mM each) and cycloheximide (250 mg/liter) with serial dilutions of the bacterial suspensions obtained from the plant rhizospheres as indicated above and were deduced from the value of the last active dilution that induced disappearance of opines in each case. This was assessed after 7 days of incubation at 28°C by high-

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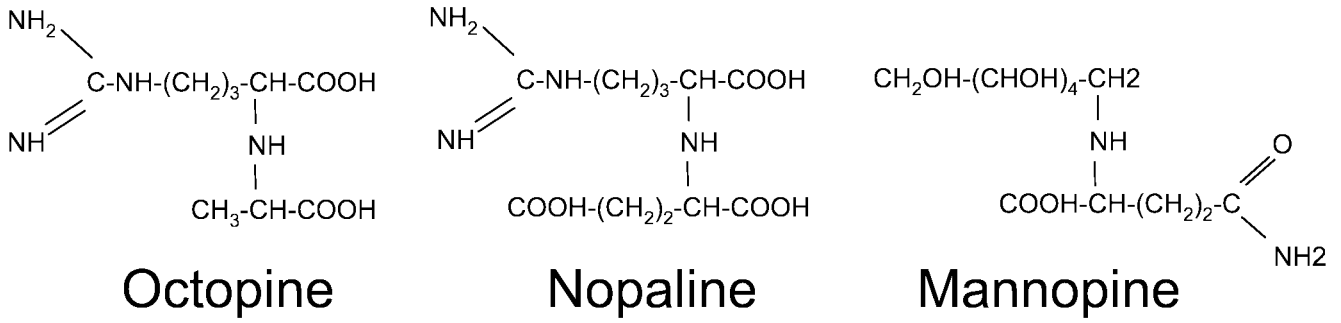

FIG. 1. Structures of opines. Octopine and nopaline are arginine and keto acid derivatives. Mannopine results from reductive condensation of glutamine and glucose (4).

voltage paper electrophoresis at pH 1.9 (4). The values presented below resulted from three independent experiments (see above), with all enumerations performed in triplicate. An analysis of variance and a Student *t* test were performed on all data collected. Values were considered significantly different at a P of 0.05.

Opine-induced bias is independent of both the opine type and the soil type. The results shown in Table 1, obtained 10 weeks after transfer of the plants to the greenhouse, indicate that the densities of the total cultivable bacteria isolated from the roots of both WT plants and opine-producing plants cultured in La-Côte-Saint-André soil did not differ significantly. A similar conclusion was drawn for the fluorescent *Pseudomonaceae* isolated from the roots of both WT plants and opine-producing plants. However, the densities of mannopine, nopaline, and octopine utilizers were 300 to 1,000 times higher in the rhizospheres of the plants producing the opines, including the previously untested compound octopine, than in the rhizospheres of the WT plants. In addition, octopine utilizers were also significantly more abundant in the rhizospheres of *Lotus* plants producing nopaline than in the rhizospheres of WT plants and plants producing mannopine (Table 1). Although not investigated, this cross-selection could be attributed to the very similar structures of the opines nopaline and octopine (Fig. 1) (for a review, see reference 4), which could therefore be degraded by the same single catabolic system in bacteria. In agreement with this hypothesis, related proteins encoded by related genes in *Agrobacterium* are involved in nopaline and octopine degradation (40, 41). In addition, proteins involved in nopaline catabolism can also use octopine as a substrate (41).

The bacterial populations colonizing the root systems of WT *Lotus* plants and of transformed *Lotus* plants harboring the oncogenes but devoid of genes encoding opine biosynthesis (ONC plants) were examined. The densities of total cultivable bacteria were identical whatever the plant of origin (WT and ONC plants) (data not shown). Similar results were obtained upon comparison of the densities of fluorescent *Pseudomonas* and the densities of opine utilizers. Consequently, the growth stimulation of opine-degrading bacteria observed around the root systems of opine-producing plants is related to expression of the opine biosynthesis genes and not to the transformed status of the plants or the presence of the pRi transferred DNA (T-DNA) oncogenes. Overall, our results indicate that the opine-dependent bias induced by transgenic, opine-producing plants also occurred with octopine-producing plants and was not specific for the soil from La Mérantaise that we used in earlier studies (21, 22). This opine-induced bias is therefore not restricted to one soil type.

Opine bias is independent of the plant species. In the second part of this study, we investigated whether the marked opine bias induced by the *Lotus* plants was specific for this plant species. We repeated the above experiments using nightshade (*S. nigrum*) plants, which are taxonomically unrelated to the genus *Lotus*, engineered to produce opines (see above). These plants were grown and transferred to the greenhouse in the La-Côte-Saint-André soil, as indicated above, and microbes

TABLE 1. Enumeration of bacterial populations from the rhizospheres of opine-producing *L. corniculatus* and *S. nigrum* in La-Côte-Saint-André soil

Bacterial group	Population size[a]						
	L. corniculatus				*S. nigrum*		
	WT plants	Plants producing mannopine	Plants producing mannopine and nopaline	Plants producing mannopine and octopine	WT plants	Plants producing mannopine and nopaline	Plants producing mannopine and octopine
Total cultivable	8.31 ± 0.06 A[b]	8.28 ± 0.08 A	8.28 ± 0.01 A	8.31 ± 0.06 A	8.14 ± 0.09 A	8.06 ± 0.03 A	8.14 ± 0.03 A
Fluorescent *Pseudomonaceae*	5.35 ± 0.10 A	5.34 ± 0.11 A	5.35 ± 0.13 A	5.34 ± 0.06 A	5.24 ± 0.08 A	5.21 ± 0.14 A	5.40 ± 0.10 A
Mannopine utilizing	3.87 ± 0.01 A	6.69 ± 0.16 B	6.88 ± 0.11 B	6.84 ± 0.08 B	3.72 ± 0.19 A	5.24 ± 0.10 B	6.53 ± 0.01 C
Nopaline utilizing	3.58 ± 0.14 A	4.07 ± 0.16 B	6.58 ± 0.14 C	4.38 ± 0.07 B	3.73 ± 0.10 A	4.91 ± 0.27 B	5.61 ± 0.21 C
Octopine utilizing	3.51 ± 0.29 A	4.16 ± 0.36 A	5.10 ± 0.17 B	6.09 ± 0.21 C	3.88 ± 0.07 A	4.95 ± 0.30 B	6.12 ± 0.26 C

[a] The values are the logarithms of the average bacterial concentrations from triplicate samples ± standard deviations.
[b] For each bacterial group, different letters after values indicate that the values are significantly different ($P < 0.05$).

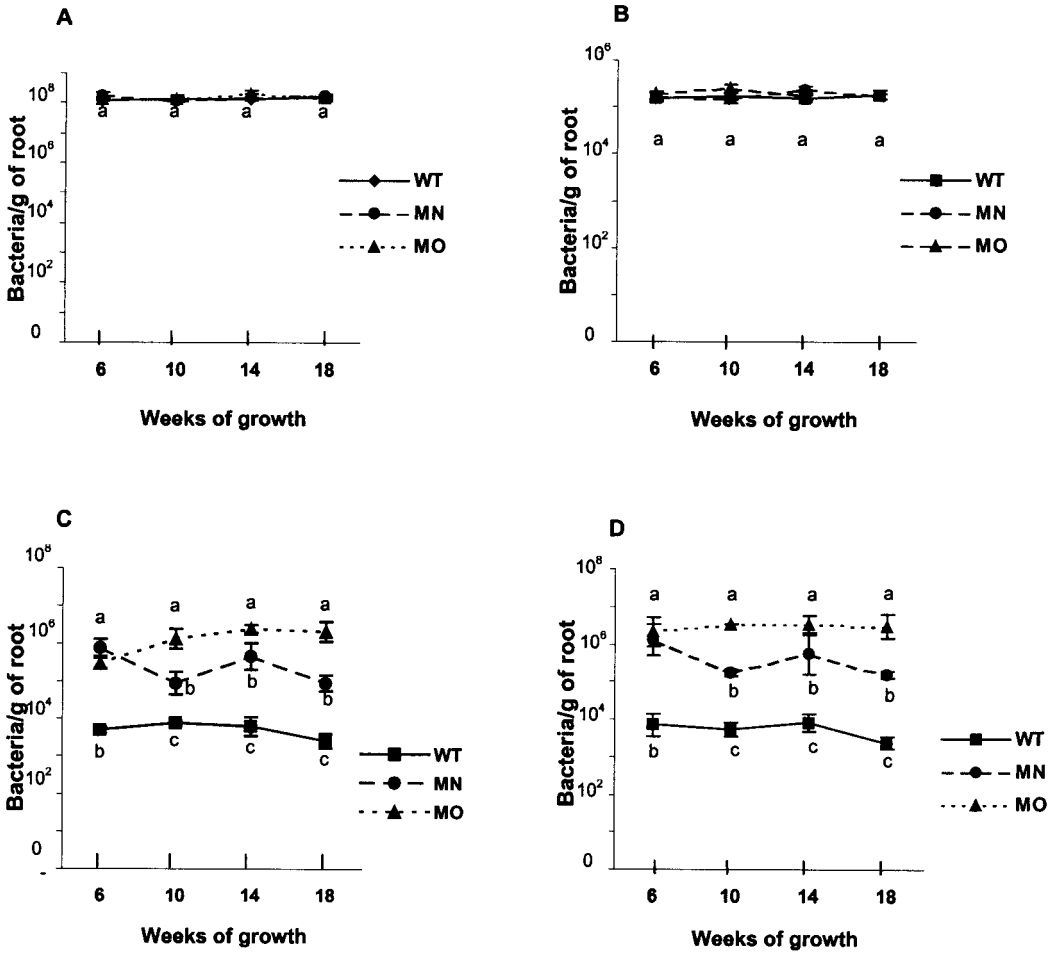

FIG. 2. Isolation over time of bacterial populations in the rhizospheres of WT *S. nigrum* (WT), *S. nigrum* producing mannopine and nopaline (MN), and *S. nigrum* producing mannopine and octopine (MO). Data points indicate average bacterial concentrations from triplicate samples, and error bars indicate standard deviations. (A) Total cultivable bacteria; (B) fluorescent *Pseudomonas*; (C) octopine-utilizing bacteria; (D) mannopine-utilizing bacteria.

associated with their root systems were analyzed as described above for the *Lotus* plants.

The results (Table 1) indicate that the densities of total cultivable bacteria isolated from the rhizospheres of the *S. nigrum* plants producing opines were identical to the densities of total cultivable bacteria isolated from the rhizospheres of the WT plants. A similar observation was made for the fluorescent *Pseudomonaceae* component of the microflora. However, as observed with the *Lotus* plants, the concentrations of mannopine-, nopaline-, and octopine-utilizing bacteria were 30 to ca. 1,000 times higher in the rhizospheres of opine-producing *Solanum* plants than in the rhizospheres of WT plants. In addition, octopine utilizers were also significantly more abundant in the rhizospheres of *S. nigrum* plants producing nopaline than in the rhizospheres of WT plants (Table 1). A comparison of the values obtained for ONC and WT plants (data not shown) suggested that the elevated densities of opine-degrading bacteria in the rhizospheres of opine-producing *S. nigrum* plants resulted from expression of opine biosynthesis genes and not from the transformed status of the plants. Additional measurements were obtained at 10, 14, and 18 weeks. The results of this series of experiments clearly indicated that the population density of total cultivable bacteria and the population density of the fluorescent *Pseudomonaceae* component of the rhizosphere were stable over the observation time (Fig. 2A and B), from 6 to 18 weeks following installation of the plants in microcosms. Furthermore, the opine-induced bias appeared to be consistently detected over time under our experimental conditions (Fig. 2C and D). Similar results have been obtained using transgenic *Lotus* plants producing opines, albeit only after 6, 10, and 14 weeks as the experiment was discontinued after 14 weeks (data not shown). This result is of interest because it has been shown previously that the microbial community selected by a plant varies according to the developmental stage of the plant (6, 16, 27), a feature that also relates to legume species (9). The apparent stability of the opine-induced bias suggests that the compositions of the root exudates of the *Lotus* and *S. nigrum* plants used in this study remained steady while the experiment lasted.

The two soil types used in our studies had different physical and chemical characteristics and originated from different geographical regions. Therefore, the microflora inhabiting these

two soils were most likely different (14, 29). As a consequence, our results obtained with the loamy soil from La-Côte-Saint-André demonstrated that the opine-induced bias generated by the opine-producing plants was not specific for a single microflora inhabiting the clay-rich soil from La Mérantaise, which was used previously (21, 22). This conclusion is supported by our results obtained with transgenic, opine-producing nightshade (*S. nigrum*) plants. Indeed, it is reasonable to assume that the microbial community inhabiting the root systems of the *Lotus* plants differed from that inhabiting the root systems of *S. nigrum* plants because the microbial communities colonizing plant roots are determined by the plant genus, species, or cultivar (3, 7, 15, 17, 28, 33, 38).

To summarize, we have shown that the effects of opine production by plants on the soil and root microflora are independent of the specific opine exuded. The data also suggest that the effect may be long term since this microbial association remained constant over the 18 weeks of the observation period. Furthermore, there are indications that these effects were also independent of plant species and soil type, but since only two different soils and two plant species were used, additional studies on these engineered associations with transformed plants are needed before definitive conclusions can be reached. These findings underline how strong the trophic perturbation brought to the rhizosphere via opine production might be. This may be attributed to the fact that opines are excellent substrates for various soil microorganisms outside the genus *Agrobacterium* (19, 36). Additionally, opines are produced at high concentrations by transgenic plants intracellularly and under hydroponic or in vitro growth conditions (8, 30, 34), and they are readily excreted. Overall, our data are in agreement with those published earlier by us and other workers and obtained in vitro and under gnotobiotic conditions (8, 31) at the leaf surface (39) or in the rhizosphere (21, 22). It is noteworthy that the stimulation ratio (ratio of the population density of opine utilizers at the surface of opine-producing plants to the population density of opine utilizers at the surface of nonproducing plants) was always much higher in studies performed with nonsterile soil, for reasons that remain to be explained.

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